Which Phenoloxidase Catalyzes Insect Cuticle Tanning, Laccase or Tyrosinase?

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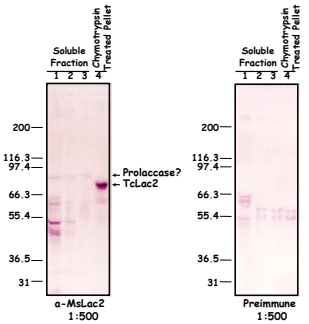
Introduction

Tanning or sclerotization is a vital process during insect development in which N-acylcatecholamines are oxidatively conjugated to cross-link proteins and stabilize the exoskeleton. The phenoloxidases laccase (Lac) and tyrosinase (Tyr) have been proposed to catalyze tanning, but evidence reported to date identifying the actual tanning enzyme has been inconclusive. To establish the involvement of either or both of these phenoloxidases in cuticle tanning, we performed RNA interference (RNAi) experiments using the red flour beetle, *Tribolium castaneum*. RNAi can be used to suppress specific messenger RNAs and generate loss-of-function phenotypes. We have knocked down phenoloxidase mRNAs and examined the phenotypes for effects on adult cuticle tanning. The results reported here demonstrate that laccase and not tyrosinase plays the major role in cuticle tanning.

Laccase is Solubilized From Pharate Adult Integument by Digestion with Chymotrypsin



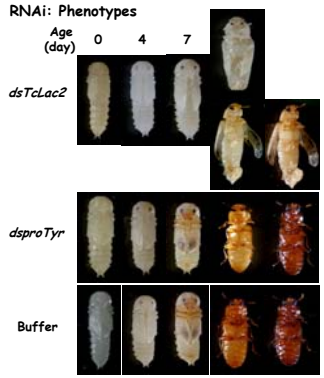
Western Blots Using MsLac2 Antibody (left) or Preimmune Serum (right)

Integuments of pharate adults were homogenized 3Xs in 100 mM sodium phosphate buffer (pH 6.5). After each homogenization step, the samples were centrifuged and the supernatants containing soluble proteins were saved for analysis (lanes 1-3). After the last homogenization, the pellet of insoluble material was then subjected to proteolysis with α-chymotrypsin in 100 mM Tris (pH 7.8), 50 mM $CaCl_2$, for 30 min at room temperature after which AEBSF was added to inhibit further proteolysis. The protease-treated sample was centrifuged and the supernatant containing chymotrypsin-solubilized proteins was saved for analysis (lane 4). Following SDS-PAGE and transfer of the proteins to a nitrocellulose membrane, duplicate blots were incubated with either polyclonal antibodies raised against the *Manduca sexta* laccase 2 protein (α-MsLac2, 1:500 dilution) or preimmune serum (1:500 dilution).

The deduced MsLac2 and TcLac2 amino acid sequences share 93% identity (unpublished data). That identity is consistent with the identities between MsLac2 and its orthologs in *Drosophila* and *Anopheles*. This relatedness supports the hypothesis that the *Manduca* laccase antibody recognizes TcLac2 and not some other unrelated proteins.

The western blotting results showed that there was an extraction buffer-soluble protein reacting with the MsLac2 antibody with an apparent molecular mass of ~85 kDa, which might be a zymogenic protein or prolaccase. The major immunoreactive protein solubilized by chymotrypsin digestion of the insoluble pellet exhibited an apparent molecular mass of ~75 kDa, which is consistent with the size expected for *Tribolium* laccase. It is likely that when tanning occurs, the laccase enzyme becomes covalently attached to the chitin-protein matrix of the exoskeleton.

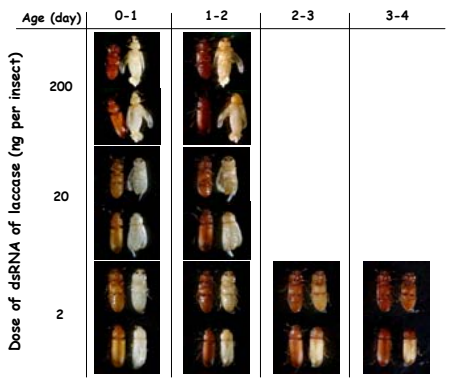
Laccase, Not Tyrosinase, Catalyzes Adult Cuticle Tanning



dsRNAs were prepared for TcLac and TcproTyr and they were designed so as to knock out both alternately spliced forms of laccase and both tyrosinase mRNAs (unpublished data). Injection of dsTcLac2 into last instar larvae caused abnormal development in all insects and generated two phenotypes. One of the phenotypes was a malformed pharate pupa that died after several days without tanning (dsTcLac2, top right). The other phenotype was a malformed, soft and expanded adult exhibiting little pigmentation It did not eclose normally and died after several days (dsTcLac2, bottom right).

Injection of either dsTcproTyr or buffer did not interfere with development or cuticle tanning.

Interference of Cuticle Tanning is Correlated with the Concentration of TcLac2 dsRNA Injected

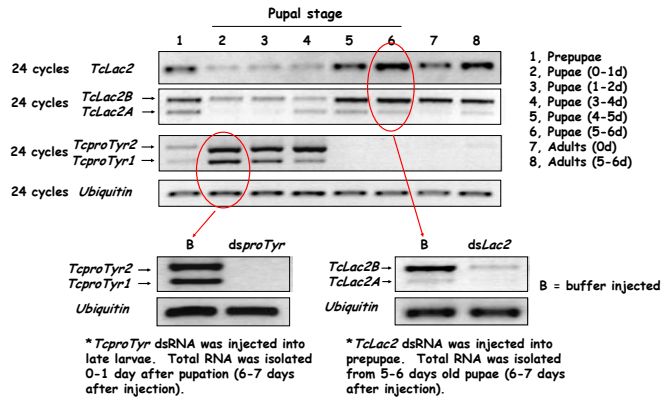


Injection of TcLac2 dsRNA into last instar larvae generated adult phenotypes with cuticle tanning inversely correlated with dsRNA concentration. Tanning was delayed by several days when 2 ng dsRNA was injected. When 20 ng was injected, tanning was almost completely prevented and the slightly malformed adults died after two days. With 200 ng, adults did not tan or eclose properly and died after 1-2 days. In each panel, the individual on the left is a control injected with buffer only and the one on the right was injected with TcLac2 dsRNA.

Developmental Pattern of Expression of Phenoloxidase Genes and RNAi of TcTyr and TcLac Genes Including Two Alternatively Spliced Forms of TcLac (2A and 2B)

Using known sequences of insect laccase and tyrosinase cDNAs, RT-PCR was used to obtain the sequences of homologous cDNAs from *Tribolium* (unpublished data). The developmental profiles of expression of *TcLac* including two alternatively spliced forms (2A and 2B) and *TcproTyr1* and *2* were determined from the prepupal through the adult stages using RT-PCR. Tyr RNAs were maximally expressed in 0-1 day old pupae, whereas Lac RNAs were highest in 5-6 day old pupae and 5-6 day old adults.

Next, we injected dsRNAs specific for each type of phenoloxidase into either late instar larvae (dsproTyr) or prepupae (dsLac), after which total RNA was isolated. mRNAs for both types of phenoloxidases were knocked down specifically by RNAi.



*TcproTyr dsRNA was injected into late larvae. Total RNA was isolated 0-1 day after pupation (6-7 days after injection).

*TcLac2 dsRNA was injected into prepupae. Total RNA was isolated from 5-6 days old pupae (6-7 days after injection).

Conclusions

1) There was a soluble laccase-immunoreactive protein with an apparent molecular mass of ~85 kDa in pharate adult cuticle, which may be a zymogenic protein or prolaccase.
2) There was an insoluble laccase-immunoreactive protein with an apparent molecular mass of ~75 kDa in pharate adult integument undergoing tanning, which probably is a *Tribolium* laccase.
3) Injection of dsRNAs specific for laccases into the body cavity of red flour beetle larvae induced the RNAi effect by knocking down laccase RNAs, produced morphological defects in adult integument, and inhibited adult cuticle tanning.
4) Injection of dsRNAs specific for tyrosinases induced the RNAi effect by knocking down tyrosinase RNAs but had no effect on adult morphology or cuticle tanning.
5) Laccase, not tyrosinase, is the primary cuticle tanning enzyme in *T. castaneum*, which oxidizes catecholic precursors of cuticular protein cross-linking agents. Tyrosinase, on the other hand, does not play a role in cuticle tanning but probably catalyzes catechol oxidations utilized in wound healing and melanization reactions during an insect immune response.

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